A MORPHOLOGICAL COMPARISON OF *HOLOSPIRA MONCLOVANA* AND *HOLOSPIRA PICTA* (GASTROPODA: UROCOPTIDAE) USING X-RAY COMPUTED TOMOGRAPHY

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ABSTRACT

This study was undertaken to re-evaluate the taxonomic placement of two species of xeric-adapted holospirids from near Monclova, Coahuila, Mexico using techniques unavailable to researchers at the time of the original descriptions of these species. X-ray computed tomography was used to scan the complete series of *Holospira monclovana* (n=24, including holotype) and of *Holospira picta* (n=27, including holotype), as well as 26 of another series of specimens (assigned to *Holospira picta* by the original author) from approximately 35 km southeast of Monclova. The program ImageJ was used to analyze X-ray CT scans of all specimens. Analyses of Similarities (ANOSIM) were conducted using the statistical suite R to compare the three populations, both pairwise and all together (Null hypothesis: $R \approx 0$; dissimilarities within groups ≥ dissimilarities between groups). Analysis shows all three populations to be statistically distinct ($R=0.398$, $p<0.001$), supports the retention of *H. monclovana* within subgenus *Holospira,* and supports reassignment of both populations of *H. picta* to the subgenus *Bostrichocentrum*.

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INTRODUCTION

This investigation re-examines the taxonomic position of three populations of desert land snails from the desert regions near the city of Monclova in the state of Coahuila in northeastern Mexico. The species under investigation are assigned to the genus *Holospira*, a little-studied group of urocoptid pulmonates first described by von Martens (1860). These xeric-adapted snails are characterized by a hollow internal axis or columella which extends from the first to the nuclear whorls; and by the cylindro-conic shape that gives them their name (*'ολοσ-,* complete, + *-σπιρα*, spire), with shells usually several times higher than their maximum width. Holospirids can be found in arid regions of the North American Southwest (including Arizona, New Mexico, Texas, and central Mexico north of central Oaxaca), usually on limestone formations (Pilsbry 1946: p 111; Thompson 1974; Thompson & Mihalcik 2005). There are several subspecies currently recognized, but "[d]istinctions between subgenera and interpretations of phylogenetic relationships are rather arbitrary because too little remains known about many of the species comprising the Holospirinae" (Thompson & Mihalcik 2005). Taxonomic decisions and revisions have historically been based on shell morphology (Gilbertson 1993), particularly the shape, number, and arrangement of certain internal ridges, or lamellae, extending into the first and second whorls (Pilsbry 1946; Thompson & Mihalcik 2005; Gilbertson & Naranjo-García 2010). These lamellae may be seen in Figure 1.

Both *Holospira monclovana* and *Holospira picta* were collected by Charles Russell Orcott and described by Paul Bartsch in the early 1920s. The two species were collected near

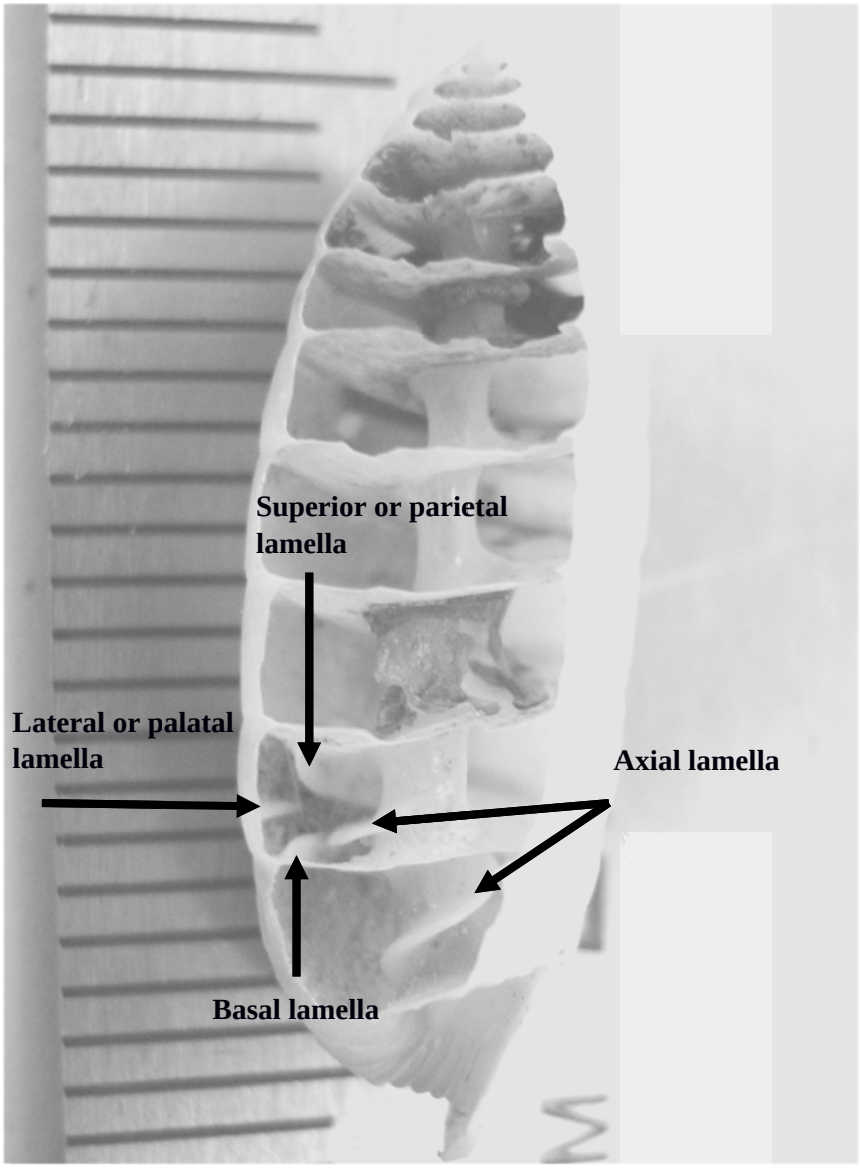

Figure 1. A representative holospirid *Holospira (Holospira) orcutti* showing the four lamellae representative of the nominate subgenus.

each other, but the type localities were imprecisely recorded—*H. monclovana* (holotype USNM 391962) at "southeast Monclova," and *H. picta* (holotype USNM 391964) "east of Monclova." A third population of snails, which Bartsch also assigned to *H. picta*, was found "at an altitude of 3000 feet, about halfway between Nueva Leon and Monclova" (Bartsch 1925). The collection from this population was stored as USNM 361966.

In addition to the holotypic specimens of *H. monclovana* and *H. picta,* Orcutt collected a series of paratypic specimens at each site. These specimens are catalogued as 361963 (*H. monclovana* paratypes), 361965 (*H. picta* paratypes, type locality), and 361966 (*H. picta* second population) in the Invertebrate Zoology collections of the United States National Museum (U.S.N.M.) of Natural History (Bartsch 1925). From the vague collection localities given the exact distance between *H. picta* type series and USNM 361966 cannot be determined, but it can be estimated at thirty to thirty-five kilometers. This range is uncharacteristically large for species of *Holospira*, most of which are restricted to areas from a few square kilometers to a few square meters (Thompson & Mihalcik 2005). Of the 76 species listed from Mexico only eight are known to have distributions beyond the immediate vicinity of their type localities (Thompson 2011).

All three populations are broadly similar in size and shape, although shells of *H. monclovana* are thicker on average than *H. picta*, and shells of the type series of *H. picta* are longer on average than *H. monclovana* (Figure 2 A-E). Curiously, the type specimen of *H. monclovana* is thicker than the average paratype specimen of *H. monclovana*, and the type specimen of *H. picta* is longer and thinner than the average paratype specimen of *H. picta* (Bartsch 1925). On average, shells of USNM 361966 are narrower and shorter than those of the other two collections (Figure 2 D-F). There is considerable overlap between the three

populations in length, width, and number of whorls: average widths and lengths vary between the two species by less than a millimeter. There is a greater difference in average length between the two populations of *H. picta* than there is between the type populations of *H. monclovana* and *H. picta*. The external differences by which Bartsch distinguishes the species appear to be based on the extent of the axial riblets, aperture shape, and coloration. *Holospira picta* has "more or less triangular," apertures while *H. monclovana* has apertures that are "broadly pear-shaped." *Holospira picta* has "a narrow zone of rusty brown immediately below the summit"; this is not mentioned for *H. monclovana* (Bartsch 1925).

Bartsch (1926) assigns *H. picta* and *H. monclovana* to two different subgenera: *H. picta* in *Eudistemma*, and *H. monclovana* in *Holospira* sensu strictu. At the time of Bartsch's classification, this would have meant different numbers of internal lamellae. *Holospira* s.s. in its adult form has four lamellae (parietal/superior, basal, axial, and palatal/lateral) in the penultimate whorl (Figure 1), while *Eudistemma* is historically defined as having a "[p]enultimate whorl with a parietal and a short axial lamella only, axis moderate" (Dall 1895). The subgenus *Stalactella* and the genus *Propilsbrya* (Bartsch 1906) also feature only these two lamellae, but the parietal lamella in both groups is characterized by many denticles. In *Stalactella* the lamellae are robust and long, while in genus *Propilsbrya* the parietal lamella extends throughout most of the spire. *Pectinistemma* (Rehder 1940) is another bilamellate genus, but both of its lamellae are serrated (Thompson & Mihalcik 2005).

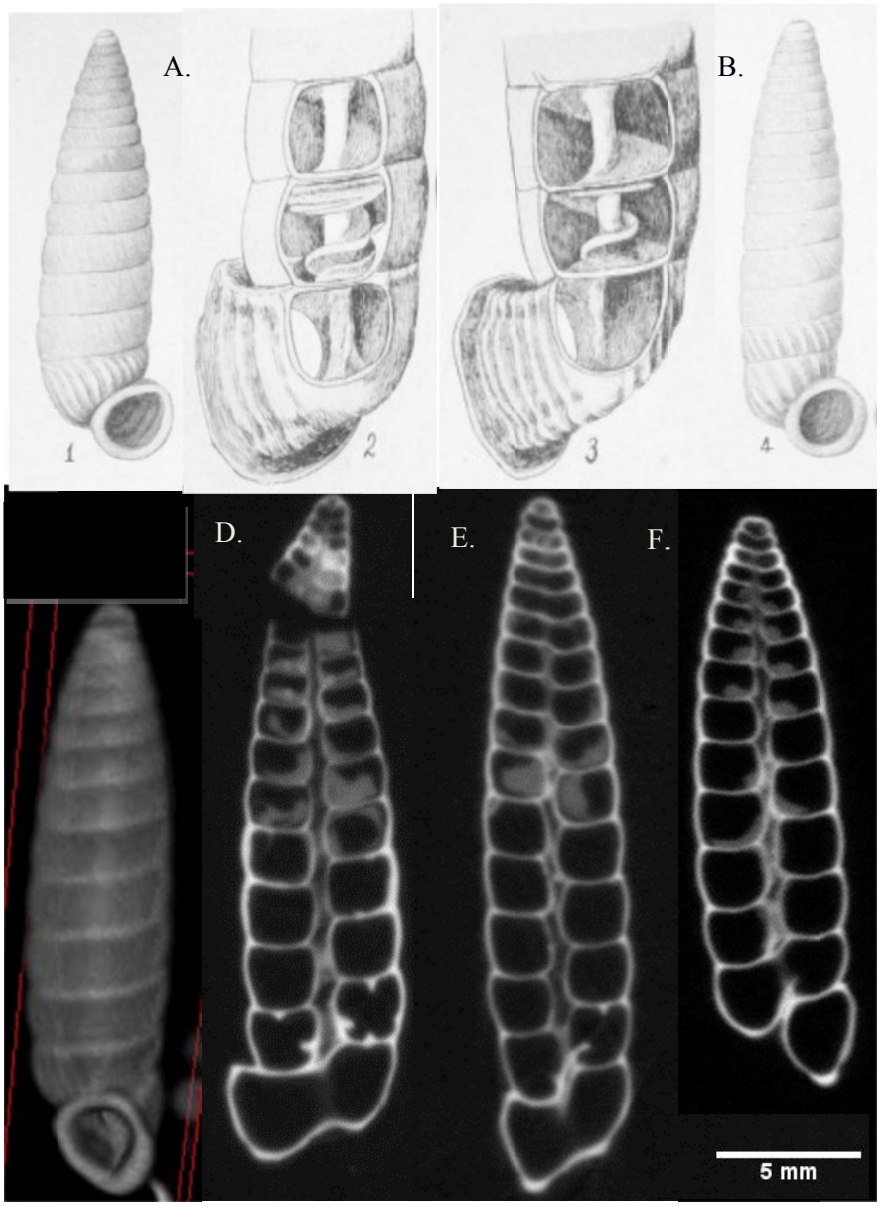

Figure 2. Internal and external views of holotype and representative specimens under investigation. (A) *Holospira monclovana* holotype; (B) *Holospira picta* holotype; (C) three-dimensional rendering of a representative specimen of USNM 361966; (D) longitudinal cross-section of *H. monclovana* holotype, (E) longitudinal cross-section of H. *picta* holotype, (F) longitudinal cross-section of a representative specimen of USNM 361966. Scale bar is for C-F. Fig. 2 A & B from Bartsch 1925. C-F are rendered by ImageJ.

This investigation is being undertaken to test the validity of Bartsch's assessment under current taxonomy, which is "in a state of flux" (Becquaert & Miller 1973). Pilsbry & Ferriss (1910: p. 117) cast doubt on using the number of lamellae forming the internal barrier as the only diagnostic criterion:

> "In several of the forms the lamellae vary from one to three, as we have demonstrated by cutting from twenty to fifty individuals of a single colony. The subgeneric distinctions (*Eudistemma, Tristemma*) based upon the number of internal lamellae in shells of this type have, therefore, no basis in nature."

Pilsbry (1946: p. 122) also states in his study of North American mollusks north of Mexico:

> "The study of a long series of many forms has shown that the lamellae in the penult whorl vary in a way it was impossible to foresee at the time when holospiras were so rare that only one or two of a lot could be opened…the number and arrangement of internal lamellae was thought at that time to be of specific and even subgeneric value. No external character is correlated with the number of lamellae."

This indicates that for some taxa, broad generalizations may have been made from one or a very few specimens. An examination of all material on loan from the USNM reveals that only a single specimen from each of the type collections, and only two specimens of USNM 361966, were opened for internal examination. Pilsbry (1946: pp 122-23) notes that different individuals from a population in the Hacheta Grande mountains could be assigned to the then-current subgenera *Tristemma, Bostrichocentrum, Haplostemma,* and *Distomospira,* while individuals in another species in the Chiricahua mountains could be categorized as

Bostrichocentrum, Tristemma, or *Eudistemma.* He ultimately defines *Eudistemma* as a synonym for *Bostrichocentrum* (Pilsbry 1946: p. 122).

Some researchers conclude that lamellar variability is restricted to select United States subgenera, and that in the other Mexican and U.S. subgenera, adult forms have reliable and consistent numbers of lamellae. Pilsbry (1946; 1953) concedes that *Holospira* s.s. contains four lamellae. Becquaert & Miller (1973) note that Mexican shells placed in *Bostrichocentrum* tend to be uniform in their lamellar arrangement, while shells from Arizona and New Mexico assigned by Pilsbry to *Bostrichocentrum* show great lamellar variation (Pilsbry & Ferriss 1910; 1915). Becquaert & Miller (1973) therefore place the "Arizona and New Mexico species" in the subgenus *Eudistemma* to distinguish them from the Mexican *Bostrichocentrum*. Thompson & Mihalcik (2005) appear to accept this for the U.S. species, although Thompson (2011) considers *Eudistemma* to be a synonym of *Holospira* s. s. when referring to Mexican holospirids, thus placing *H. picta* in the subgenus Holospira. According to Thompson & Mihalcik (2005), Mexican shells lacking a fully developed lamellar array tend to be "atypically thin"; during definitive development the columellar lamellae develop first, followed by the parietal, basal, and finally palatal lamellae.

Gilbertson (1993) distinguishes *Eudistemma* from *Bostrichocentrum* (sensu Becquaert & Miller 1973) by anatomical differences in the epiphallus and penis of specimens with soft tissues preserved. Unfortunately, intact tissue specimens of *H. monclovana* and *H. picta* are unknown. Many holospirids were described long before it was customary to take tissue samples, so the type specimens or type series are preserved only as shells. As many species have extremely restricted distributions (Becquaert & Miller 1973; Thompson &

Mihalcik 2005) living specimens may be difficult to find if the type locality was poorly described or if anthropogenic activity has encroached upon their original habitats.

Current taxonomic weighing of characters supports the premise that the nominate subgenus is found in Mexico and parts of the southeastern United States (Pilsbry 1946; 1953; Thompson & Mihalcik 2005; Thompson 2011) while *Bostrichocentrum* (Strebel & Pfeiffer 1880) is restricted to Mexico (Thompson 2011) and has one axial lamella, or rarely an axial and a parietal lamella (Pilsbry 1953). *Stalactella* is found in the Puebla and Oaxaca area of México and has a long axial and a denticulate parietal lamella (Thompson & Mihalcik 2005). *Eudistemma* is confined to the southeastern United States and has variable numbers of lamellae (Becquaert & Miller 1973). *Holospira* s. s. has four lamellae ancestrally, although trilamellate species may be included if the lamellae are nonserrated (Gilbertson & Naranjo-García 2010). The other subgenera in the genus *Holospira* are beyond the scope of this investigation.

This investigation uses noninvasive scanning methods and statistical analyses to determine the subgeneric assignment of *H. picta*. Other questions under investigation are whether or not *H. picta* is significantly different from *H. monclovana*; and whether or not the two populations assigned to *H. picta* are indeed the same species.

In the absence of soft tissues or well-preserved genetic samples, data based on shell morphology must be used. Morphology can uncover relationships between species (Zelditch et al. 2000), and has been successful in clarifying relationships of gastropods (Perez & Strenth 2003). While genetic data is often preferable, recent thorough searches of the Monclova area have failed to produce any living specimens of either species (Strenth, pers. comm.). Living specimens are unknown for many described species of *Holospira*—several

are known only from shell material. This investigation demonstrates a method by which internal features may be mathematically compared without using live specimens or destroying museum specimens. This technique should be useful when dealing with species for which soft tissues or genetic material is unavailable (including species extinct in the wild) and may help contribute to the ongoing endeavor to weigh taxonomic characters of the genus properly.

MATERIALS AND METHODS

Material examined.—The entire series of *H. monclovana* and *H. picta,* including holotype and paratypes, were obtained from the USNM, as was the entire collection of USNM 361966.

Holospira monclovana—This analysis included 24 specimens: the holotype specimen (USNM 361962) and 23 of the 24 specimens from the paratypic series (USNM 361963). One specimen was missing its aperture and part of the first whorl and was excluded from analysis. The holotype specimen was broken at the tenth whorl but both pieces were present, allowing total height and columellar height to be measured additively.

Holospira picta—Analysis included 27 specimens: the holotype specimen (USNM 361964) and 26 of the 31 specimens in the paratypic series (USNM 361965). Five paratype specimens were missing the aperture and parts of one or more whorls, and were thus excluded from analysis.

USNM 361966, labeled as *H. picta*—Analysis included 26 specimens from a lot of 500+. These 26 were in three vials separate from the box containing the main lot, and presumably are the specimens mentioned and measured in the original description (Bartsch 1925), as they contain two partially sectioned specimens.

Scanning procedures.—Such rare specimens could not be physically sectioned, so this investigation employed completely noninvasive high-power X-ray computed tomography (CT). This technique provides information about the internal features of practically any structures with differing internal densities (Carlson et al. 2003), including snail shells (Postnov et al. 2002). X-ray CT takes a series of 2-dimensional views through an object which can then be compiled into a 3-D image. Measurements can be taken from the 3-D

image rendered, or simply on the individual 'slices' or frames. A preliminary investigation conducted on *Holospira orcutti,* a member of subgenus Holospira from near Paredon, México, has shown CT scanning process to be effective on holospirid shells. The resulting scans are adequate in resolution for study purposes, and accurate measurements can be taken from them (Rilling & Strenth 2010). The specimens obtained were hand-delivered to the CT lab at University of Texas at Austin and scanned. The specimens were returned unharmed to the USNM.

For high resolution, the scanner was in high-power mode with the intensity control off. Each x-ray 'slice' was 0.054 mm thick; slices were taken every 0.054 mm to avoid overlap and ensure complete coverage. A field of reconstruction 5 cm in diameter was enough to include 24-26 specimens in a given series—the smaller the field of reconstruction, the greater the resolution. The holotype specimens were scanned separately from the paratype series. A series of five hundred transverse sections was sufficient to scan the entire length of the longest shell; excess sections were deleted from the data, yielding 478 cross-sections of the *H. monclovana* holotype, 447 cross-sections of *H. monclovana* paratypes*,* 454 cross-sections of the *H. picta* holotype, 470 cross-sections of *H. picta* paratypes, and 419 cross-sections of USNM 361966. Sections were saved as 16-bit TIFF images. The image manipulation tool ImageJ 1.45s (Abramoff et al. 2004) was used to compile the cross-sections into 'stacks' which were 'resliced' to yield secondary longitudinal sections for each individual shell.

Measurements.—A series of 23 linear and angular parameters was measured from the individual scans (Table 1) using the ImageJ program. The angular measurements (Characters 1-4) and Characters 13, 20, 22, and 23 were measured on the cross-sectional sections;

Table 1. List of shell measurements recorded using ImageJ.

No.	Character	Description of character
1.	axial	Rotation of axial lamella, in degrees
2.	superior	Rotation of superior/ parietal lamella, in degrees, if present
3.	basal	Rotation of basal lamella, in degrees, if present
4.	palatal	Rotation of palatal/lateral lamella, in degrees, if present
5.	total height	Total height of the shell, from the top of spire to the bottom of the aperture lip
6.	columella height	Distance from the first appearance of space between columella walls to the opening of the columella.
7.	1st height	Height of first whorl from lowest point of aperture insertion to suture
8.	2nd height	Height of second whorl from suture to suture on the side opposite aperture opening
9.	3rd height	Height of third whorl from suture to suture on the side opposite aperture opening
10.	diameter	Maximum diameter of shell between second and sixth whorls
11.	columella width	Maximum width of the columella from internal wall at suture to external wall opposite
12.	col. width oblique	Maximum width of columella between sutures, from external wall to external wall perpendicular to columella axis
13.	col. x. section	Maximum width of columella from external wall to external wall measured on cross-sectional scan
14.	col. internal	Maximum internal diameter of columella from one internal wall to the other at a suture point
15.	axial height	Maximum height of axial lamella
16.	superior height	Maximum height of superior/parietal lamella
17.	basal height	Maximum height of basal lamella
18.	palatal height	Maximum height of palatal/lateral lamella
19.	aperture height	Aperture interior depth
20.	aperture width	Aperture interior maximum width
21.	lip bottom	Lip length at bottom of aperture
22.	lip palatal	Greatest lip length at palatal/lateral edge of aperture
23.	lip medial	Greatest lip length at medial edge of aperture

while Characters 5-12, 14, 16, 17, 19, and 21 were measured on longitudinal sections. Characters 15 and 17 were measured on both section types and the mean value recorded.

As many simple linear measurements correlate with overall size, characters 6-10 and 14-23 were adjusted by dividing them by the total shell length. The mean of the columellar width characters 11-13 was taken for each shell and the resulting values were divided by total shell length (Table 2). Note that, as all specimens in the investigation possessed an axial lamella, 'presence of axial lamella' was not included in the analysis as it would not have yielded any variation within or between groups.

Analysis.—The measurement data were used to create a triangular dissimilarity matrix for all specimens under study in the three populations of snails, using the Bray-Curtis dissimilarity coefficient. The Bray-Curtis coefficient is widely used for generating dissimilarity matrices from multivariate data, as it is resistant to scale changes, more robust than Euclidean distance measures, and broadly reliable (Clarke 1993). An ordination of dissimilarities for each specimen was generated using Kruskal's non-metric multi-dimensional scaling (NMDS) to rank the dissimilarities between specimens. NMDS compares well to other methods of ordering complex multivariate data and requires fewer assumptions than principal components analysis or correspondence analyses (Kenkel & Orlóci 1986; Clarke 1993).

Ordination data can be graphically represented on a plot with one or more axes; ideally the dimensionality or number of axes will be small (Kenkel & Orlóci 1986). In this analysis a two-dimensional plot was sufficient to illustrate almost all of the dissimilarity. In an ideal NMDS plot, distance between points and dissimilarities between the items they represent are in a 1:1 linear relationship; i.e., specimens that are the most dissimilar are the

farthest apart on the plot. While the plot itself does not test an hypothesis, it is valuable in showing relationships of complex data sets (Clarke 1993).

An analysis of similarities, or ANOSIM, was then performed on the ordination data. This type of analysis has historically been used in community ecology to compare community compositions at different sites (Clarke 1993), but is useful for many kinds of multivariate datasets. A series of spatial measurements for each of a number of shells within a population can be thought of as analogous to a series of species abundance measurements for each of a number of sites marked by a particular environmental variable. ANOSIM is a non-parametric test of difference between groups, comparing dissimilarities between groups to dissimilarities within groups. It is similar in some ways to an analysis of variance on the dissimilarity matrix, but instead of using the ratio of sums of squares like the F-statistic generated by ANOVA, ANOSIM generates an R-statistic based on mean ranks of dissimilarities within and between groups.

The null hypothesis is that there is no difference between the three snail populations; i.e., that the R-statistic calculated will not be significantly different from zero. The alternate hypothesis—if the R-statistic is significantly different from zero—is that there is a difference between populations. Pairwise ANOSIM tests were conducted between each pair of groups as a post-hoc test.

A more robust (Oksanen et al. 2011) permutational MANOVA analogue, ADONIS, was also conducted to analyze the Bray-Curtis dissimilarity matrix. ADONIS differs from MANOVA in conducting F-tests on sums of squares using permutations of the raw data rather than residuals. It also does not demand the assumptions required by the standard MANOVA. ADONIS analysis does not generate an ordination plot.

These analyses were conducted using the statistical software R, version 2.14.1 (R.D.C.Team, 2008) and the 'vegan' analysis package (Oksanen et al. 2011).

Table 2. List of characters used for analysis. Asterisks denote characteristics that could not be included in the ANOSIM dissimilarity matrix due to values being absent in some groups and present in others.

Character	Description of character
ax	Rotation of axial lamella, in degrees
sup	Rotation of superior/ parietal lamella, in degrees
bas*	Rotation of basal lamella, in degrees
pal*	Rotation of palatal/lateral lamella, in degrees
pressup	Presence or absence of superior lamella
presbas	Presence or absence of basal lamella
prespal	Presence or absence of palatal lamella
totalht	Total height of the shell, from the top of spire to the bottom of the aperture lip
adjcolht	Ratio of the length of the columella to total height
adj1stht	Ratio of height of first whorl (from lowest point of aperture insertion to suture) to total height
adj2ndht	Ratio of height of second whorl (from suture to suture on the side opposite aperture opening) to total height
adj3rdht	Ratio of height of third whorl (from suture to suture on the side opposite aperture opening) to total height
adjdiam	Ratio of maximum diameter to total height
adjcolwd	Ratio of columellar maximum width to total height
adjcolint	Ratio of maximum internal diameter of columella (from one internal wall to the other) to total height
adjaxht	Ratio of maximum height of axial lamella to total height
adjsupht	Ratio of maximum height of superior/parietal lamella to total height
adjbasht*	Ratio of maximum height of basal lamella to total height
adjpalht*	Ratio of maximum height of palatal/lateral lamella to total height
adjapht	Ratio of aperture interior depth to total height
adjapwd	Ratio of aperture interior maximum width to total height
adjlipb	Ratio of lip length at bottom of aperture to total height
adjlipp	Ratio of greatest lip length at palatal/lateral edge of aperture to total height
adjlipm	Ratio of greatest lip length at medial edge of aperture to total height

RESULTS

Observational.—Upon examination, all specimens of *H. monclovana* proved to exhibit all four lamellae which are characteristic of the nominate subgenus. All specimens of USNM 361966 and all but one specimen of *H. picta* displayed an axial lamella, and most displayed a parietal lamella as well. The *H. picta* specimen lacking the axial lamella also had the aperture and part of the first whorl missing and was thus excluded from the analysis. Nine of the *H. picta* specimens (25%) and seven of the USNM 361966 specimens (27%) examined lacked a parietal lamella. Lamellae were uniformly nonserrated and toothless.

Specimens of *H. monclovana* proved to be more robust than the other two populations in terms of lamellar height, lamellar rotation, columellar width, and aperture size. Specimens of *H. picta* tended to be longer on average than the other two populations. USNM 361966 was the shortest and narrowest of the three, and also had the least extensive lamellation and the smallest aperture. It did, however, have more extensive aperture lips on average than did the other two populations (Table 3).

Statistical.— The Analysis of Similiarities showed that dissimilarities between groups were greater than dissimilarities within groups ($R=0.3977$, $p<0.001$) when considering all three populations (Figure 3 A). Pairwise post-hoc ANOSIM tests revealed that dissimilarities between groups were greater than within groups for each pair of populations (Figure 3 B-D) *H. monclovana* is distinct from *H. picta* ($R=0.612$, $p<0.001$) and USNM 361966 ($R=0.466$, $p<0.001$); and *H.picta* and USNM 361966 are distinct ($R=0.098$, $p<0.006$) (Appendix A).

Table 3. Mean values of measurements taken for each population.

No.	Character	*H. monclovana*	*H. picta*	USNM 361966
1.	axial (°)	417	288	230
2.	superior(°)	350	131	115
3.	basal (°)	114	N/A	N/A
4.	palatal (°)	156	N/A	N/A
5.	total height (mm)	19.93	20.13	18.7
6.	col. height (mm)	16.72	17.52	16.06
7.	1st height (mm)	3.11	3.06	2.92
8.	2nd height (mm)	1.9	1.82	1.74
9.	3rd height (mm)	1.88	1.81	1.72
10.	diameter (mm)	5.46	4.71	4.55
11.	col. width (mm)	0.9	0.63	0.67
12.	col. width obl. (mm)	0.91	0.65	0.67
13.	col. x. section (mm)	0.92	0.66	0.72
14.	col. internal (mm)	0.69	0.44	0.46
15.	axial height (mm)	0.68	0.38	0.41
16.	superior height (mm)	0.76	0.16	0.12
17.	basal height (mm)	0.39	N/A	N/A
18.	palatal height (mm)	0.42	N/A	N/A
19.	aperture height (mm)	2.64	2.52	2.37
20.	aperture width (mm)	2.66	2.4	2.27
21.	lip bottom (mm)	0.52	0.48	0.62
22.	lip palatal (mm)	0.52	0.51	0.67
23.	lip medial (mm)	0.49	0.44	0.56

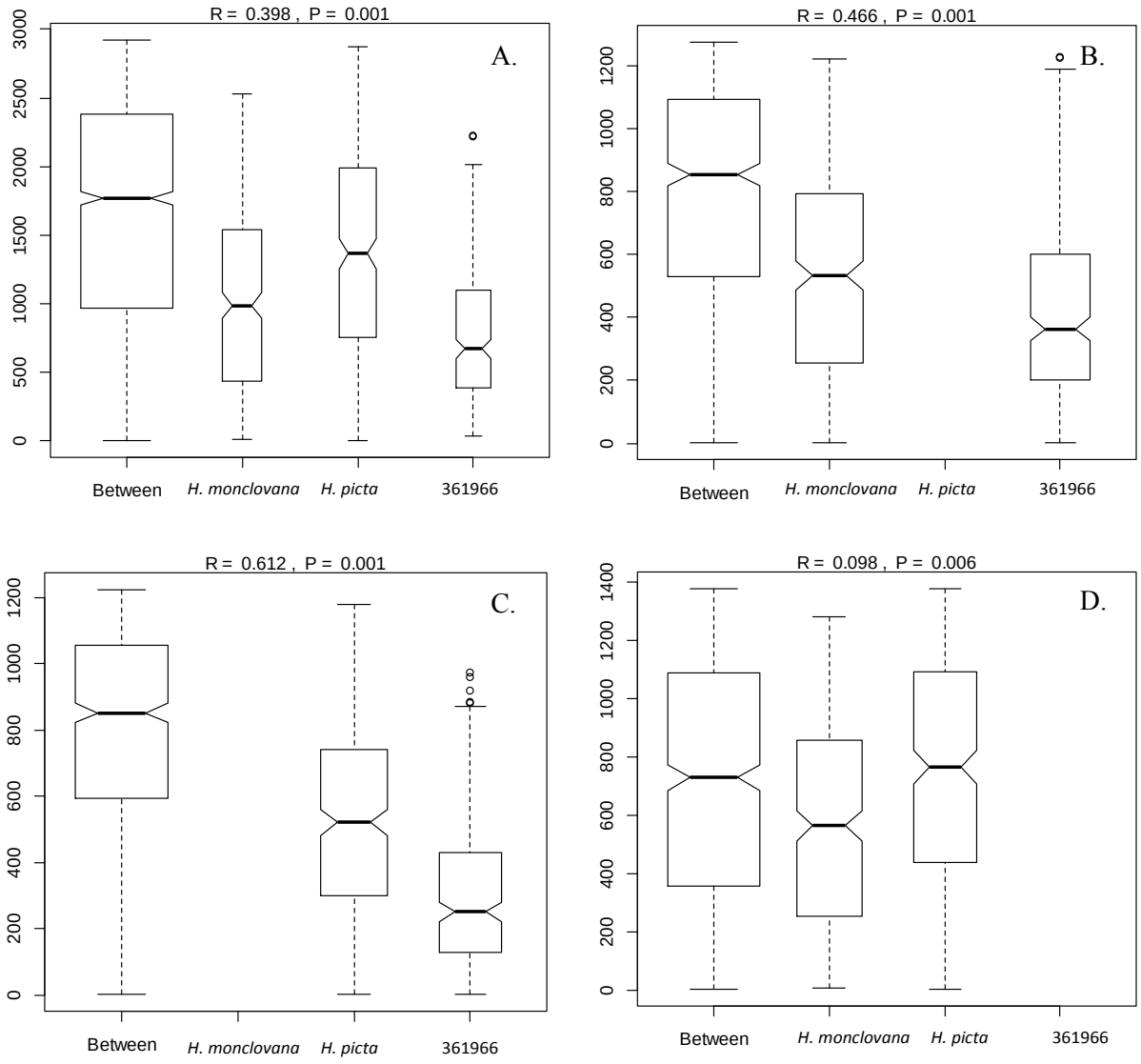

Figure 3. ANOSIM box-and-whisker plots of dissimilarities between and within groups (A) comparing all three populations, (B) comparing *H. monclovana* and USNM 361966, (C) comparing *H. monclovana* and *H. picta,* (D) comparing *H. picta* and USNM 361966. Notches in the boxes are analogous to 95% CI. Y-axes are unitless measures of dissimilarities.

The ordination plot of the dissimilarity matrix (Figure 4) did not show perfect separation between populations, although the *H. monclovana* specimens tended to cluster in the middle left-hand section of the diagram while USNM 361966 specimens tended to confine themselves to the right-hand side of the diagram (Table 4). The reliability of the NMDS plot is checked with a Shepard diagram, a scatterplot of distances in the dissimilarity matrix versus distances on the NMDS plot. The less the total scatter, the better the NMDS plot conforms to the data in the dissimilarity matrix (Clarke 1993). The R^2 values for the best-fit regression lines were 0.988 for a nonmetric fit and 0.954 for a linear fit (Figure 5).

Factors contributing to the multidimensional scaling axes can be seen in Figure 6. The greatest contributing factor to the x-axis (labeled MDS1) is the rotation of the axial lamella. Other factors that contributed significantly to the axes, in descending order of R^2 values include presence of basal and palatal lamellae, adjusted axial lamellar height, adjusted columellar internal width, total height, adjusted columellar width, adjusted diameter, adjusted columellar height, adjusted height of the second whorl, adjusted aperture height and width, presence of a superior/parietal lamella, and adjusted third whorl height. Neither the adjusted first whorl height nor any of the aperture lip measurements contributed significantly to the NMDS axes (Table 5).

Confidence ellipses were drawn on the NMDS plot for 95% and 99% confidence intervals. It can be seen that the multivariate centroid for *H. monclovana* is well-separated from the other two populations. The 95% (inner) confidence ellipses of *H. picta* and USNM 361966 do not overlap, but their 99% (outer) confidence ellipses do, the 99% ellipse of USNM 361966 overlapping even a portion of *H. picta's* 95% confidence ellipse (Figure 7).

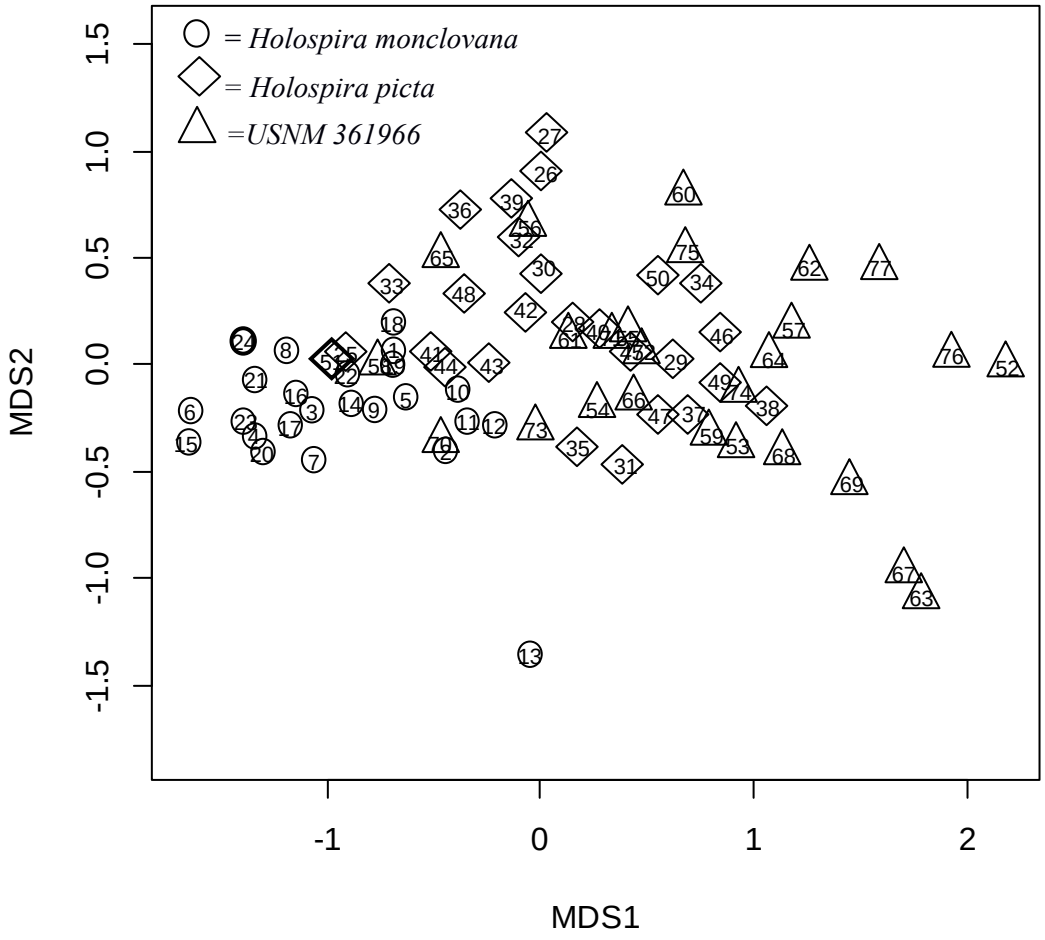

Figure 4. Ordination plot of the dissimilarity matrix using nonmetric multidimensional scaling (NMDS). Specimens 1 through 24, in circles, represent *H. monclovana*. Specimen 24 (bold circle) is the holotype of *H. monclovana*. Specimens 25 through 51, in lozenges, represent *H. picta*. Specimen 51 (bold lozenge) is the holotype of *H. picta*. Specimens 52 through 77 represent USNM 361966.

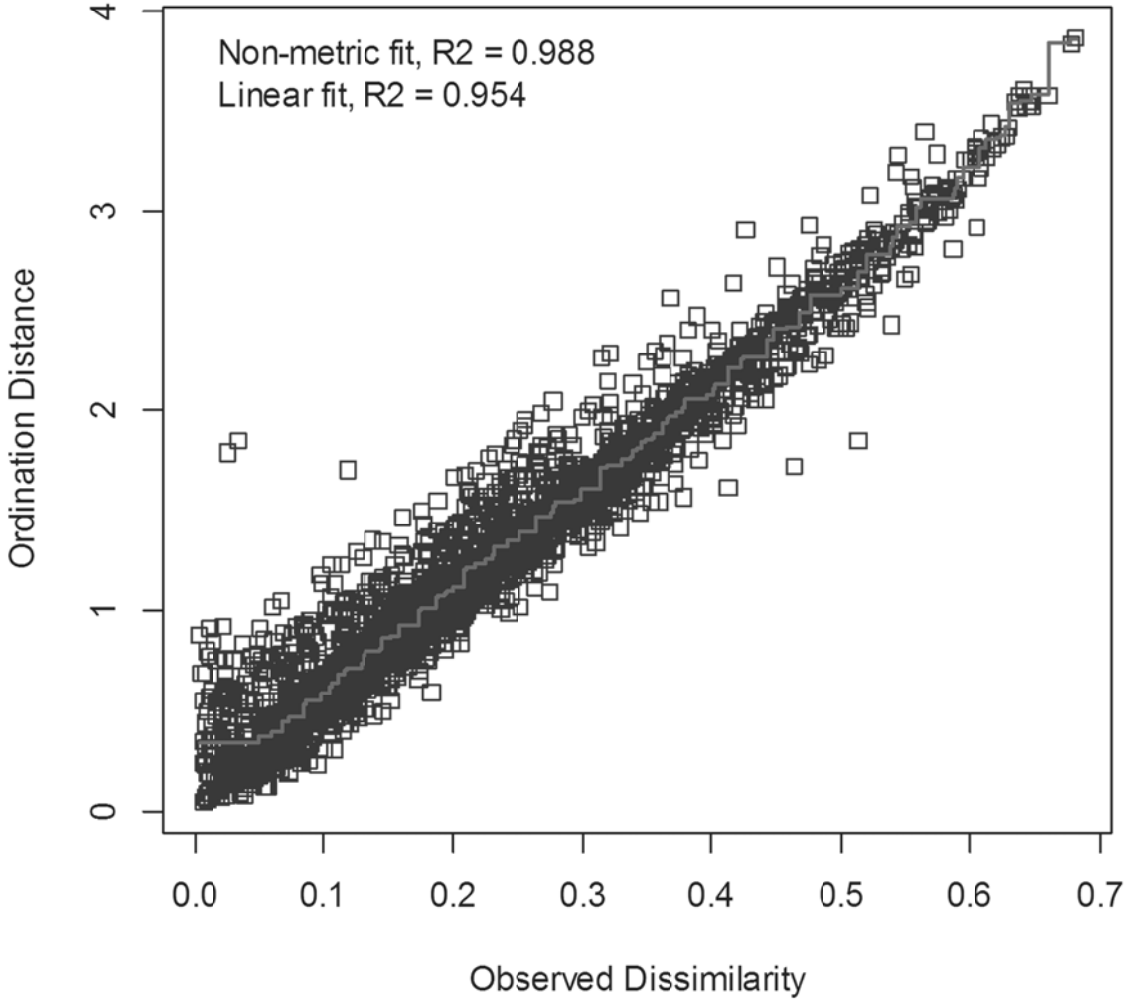

Figure 5. Shepard diagram of distances in the dissimilarity matrix against distances in the NMDS ordination plot used in Figs. 3, 6, and 7. Scatter around the best-fit regression line indicates 'stress' (discrepancy between rank-order distances and distances in the NMDS plot).

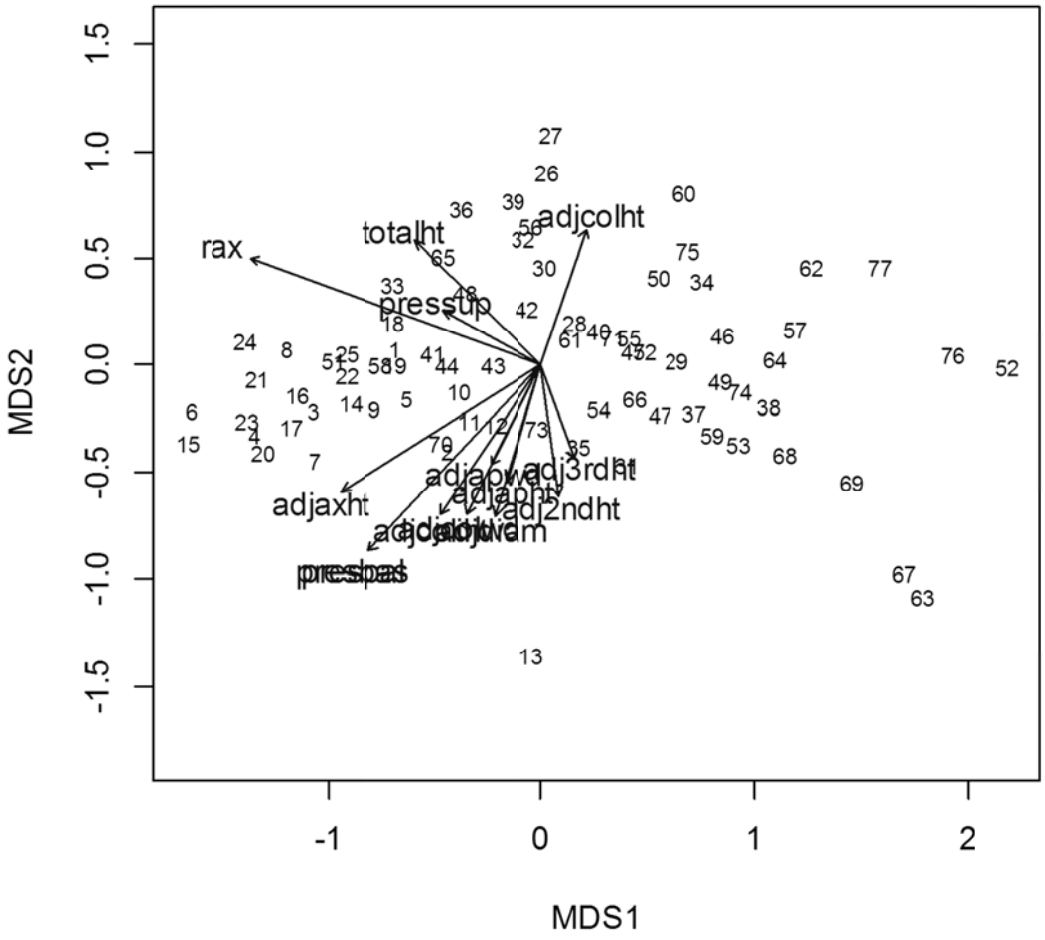

Figure 6. NMDS plot displaying character vectors contributing to the multidimensional axes. See Tables 2 and 5 for further information on the extent and contribution of each vector.

Table 4. Dissimilarity test scores for multidimensional scaling calculated using R 2.14.1. Scaling based on 999 permutations.

No.	Type	MDS1	MDS2
1	*H. monclovana*	-0.68446240	0.085508052
2	*H. monclovana*	-0.44706618	-0.397339286
3	*H. monclovana*	-1.07180336	-0.207444350
4	*H. monclovana*	-1.34189506	-0.319386162
5	*H. monclovana*	-0.62658148	-0.152081936
6	*H. monclovana*	-1.64619999	-0.202476159
7	*H. monclovana*	-1.05653473	-0.444074341
8	*H. monclovana*	-1.19004703	0.083776643
9	*H. monclovana*	-0.77563874	-0.197173343
10	*H. monclovana*	-0.39090448	-0.114927692
11	*H. monclovana*	-0.34200045	-0.257370268
12	*H. monclovana*	-0.21383874	-0.275176427
13	*H. monclovana*	-0.04576497	-1.345902601
14	*H. monclovana*	-0.88911507	-0.168944762
15	*H. monclovana*	-1.66375574	-0.356889574
16	*H. monclovana*	-1.14898655	-0.127287944
17	*H. monclovana*	-1.17144586	-0.283284355
18	*H. monclovana*	-0.69130369	0.201084183
19	*H. monclovana*	-0.68663981	0.006366910
20	*H. monclovana*	-1.30266389	-0.400636416
21	*H. monclovana*	-1.33505706	-0.057801868
22	*H. monclovana*	-0.90789118	-0.037544919
23	*H. monclovana*	-1.38108680	-0.254151665
24	*H. monclovana*	-1.39193143	0.117646574
25	*H. picta*	-0.91504552	0.063318532
26	*H. picta*	0.02524471	0.911871203
27	*H. picta*	0.04290115	1.084212587
28	*H. picta*	0.15201657	0.207775703
29	*H. picta*	0.63452665	0.030475239
30	*H. picta*	0.01555109	0.463277018
31	*H. picta*	0.39908458	-0.460960041
32	*H. picta*	-0.08347871	0.596863148
33	*H. picta*	-0.69507723	0.387034343
34	*H. picta*	0.75552901	0.398168844
35	*H. picta*	0.17199755	-0.374781921
36	*H. picta*	-0.37593987	0.741093065
37	*H. picta*	0.71857406	-0.211747895
38	*H. picta*	1.06426019	-0.184007325

Table 4. Continued

No.	Type	MDS1	MDS2
39	*H. picta*	-0.13290131	0.777136893
40	*H. picta*	0.27296647	0.166059875
41	*H. picta*	-0.50483323	0.066003984
42	*H. picta*	-0.06421722	0.270427178
43	*H. picta*	-0.22782736	0.004702418
44	*H. picta*	-0.44510398	0.006288231
45	*H. picta*	0.42746455	0.077741556
46	*H. picta*	0.85302413	0.149039747
47	*H. picta*	0.56383607	-0.227213480
48	*H. picta*	-0.35732235	0.343111251
49	*H. picta*	0.84035665	-0.067436658
50	*H. picta*	0.54764557	0.416904705
51	*H. picta*	-0.97422761	0.028439571
52	USNM 361966	2.18696210	-0.005131441
53	USNM 361966	0.92617227	-0.367927365
54	USNM 361966	0.27367715	-0.193998090
55	USNM 361966	0.40618288	0.136941351
56	USNM 361966	-0.05071792	0.652279488
57	USNM 361966	1.18972990	0.179552965
58	USNM 361966	-0.74836978	0.011738938
59	USNM 361966	0.80544841	-0.323671131
60	USNM 361966	0.66814789	0.816029932
61	USNM 361966	0.14126089	0.130479866
62	USNM 361966	1.26563891	0.469530323
63	USNM 361966	1.78733095	-1.081942289
64	USNM 361966	1.09319734	0.036857156
65	USNM 361966	-0.46218835	0.516130515
66	USNM 361966	0.44237888	-0.146558955
67	USNM 361966	1.70312289	-0.960883651
68	USNM 361966	1.14436992	-0.408274757
69	USNM 361966	1.45710486	-0.541653667
70	USNM 361966	-0.47256414	-0.357634867
71	USNM 361966	0.33760804	0.143955740
72	USNM 361966	0.48626342	0.077228672
73	USNM 361966	-0.02533945	-0.292554592
74	USNM 361966	0.93289327	-0.115172836
75	USNM 361966	0.68671341	0.545392553
76	USNM 361966	1.92504906	0.057686408
77	USNM 361966	1.59353730	0.463313664

Table 5. List of vectors and their contributions to the axes of the ordination plot calculated using R 2.14.1. Significant vectors are included on Figure 6. Significance codes: ***= p < 0.001; **=p < 0.01; *= p< 0.05.

	MDS1	MDS2	r2	Pr(>r)
ax	-0.93803	0.34655	0.8955	0.001 ***
pressup	-0.87179	0.48989	0.1165	0.007 **
presbas	-0.67856	-0.73454	0.5955	0.001 ***
prespal	-0.67856	-0.73454	0.5955	0.001 ***
totalht	-0.71271	0.70146	0.2901	0.001 ***
adjapht	-0.28858	-0.95746	0.1370	0.004 **
adjapwd	-0.45293	-0.89155	0.1165	0.007 **
adjlipb	0.70591	-0.70830	0.0070	0.806
adjlipp	0.40072	0.91620	0.0312	0.323
adjlipm	0.54770	-0.83668	0.0119	0.669
adjcolht	0.31878	0.94783	0.1899	0.001 ***
adjdiam	-0.29382	-0.95586	0.2298	0.001 ***
adj1stht	0.10184	-0.99480	0.0240	0.402
adj2ndht	0.13582	-0.99073	0.1613	0.001 ***
adj3rdht	0.33443	-0.94242	0.0927	0.017 *
adjcolwd	-0.45180	-0.89212	0.2589	0.001 ***
adjcolint	-0.56009	-0.82843	0.3022	0.001 ***
adjaxht	-0.84524	-0.53439	0.5205	0.001 ***

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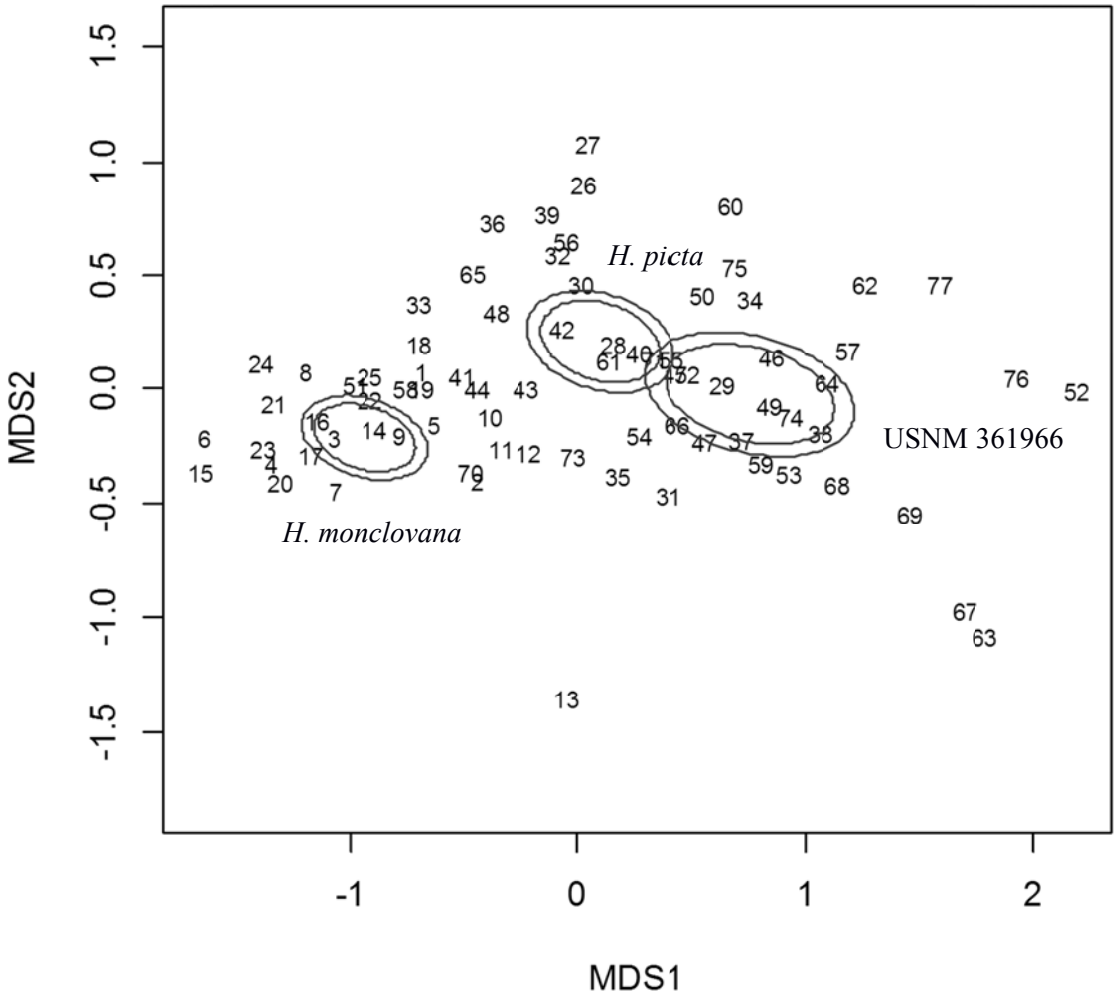

Figure 7. NMDS plot showing 95% (inner) and 99% (outer) confidence ellipses for each population of snails. Ellipses are centered on multivariate centroids of each population.

Results of the ADONIS analysis were similar to the ANOSIM test. The population to which a snail was assigned did have a significant effect ($R=0.506$, $p<0.001$, Bray-Curtis dissimilarity, 999 permutations). Post-hoc pairwise comparisons again confirmed USNM 361966 and *H. picta* were significantly distinct ($R=0.153$, $p<0.0016$, Bray-Curtis dissimilarity, 9999 permutations), although only 15.3% of the variance between the two populations was accounted for by type (Appendix B).

DISCUSSION

The definition of subgenus *Holospira* s. s. has not changed since Bartsch's time, and the consistency in the numbers of internal lamellae support the assignment of *H. monclovana* to the nominate subgenus. Both *Holospira picta* and USNM 361966 have an axial and usually a parietal lamella and are clearly distinct from *H. monclovana*. The reliable presence of the small, toothless axial lamella is consistent with the subgenus *Bostrichocentrum,* and given the location, *H. picta* and USNM 361966 are unlikely to be *Eudistemma* sensu Becquaert and Miller 1973. (It is noteworthy that Dall's 1895 definition of *Eudistemma* as "parietal and a short axial lamella only" fits perfectly)

The occasional absence of the parietal lamella in both *H. picta* and USNM 361966 may be due to incomplete ontogenic development (Thompson & Mihalcik 2005), although the shells missing the parietal lamella are by no means always the smallest. Thin walls and missing lamellae could be as readily explained by low calcium reserves as by developmental stage, and would fit at least as well with the size data.

USNM 361966 is similar to *H. picta,* but smaller in most dimensions except for aperture lip length. Previous research on land snails, including xeric land snails from the Middle East and Texas, has found correlations between geography/climatic conditions and shell shape (Nevo et al. 1981, Nevo et al. 1983, Perez 2001). In at least two species of Negev desert snails in the genus *Xerocrassa,* maximum diameter correlates positively with humidity and negatively with evaporation/aridity; this is presumed to minimize water loss by decreasing aperture size (Nevo et al. 1981). On the other hand, some species of *Sphincterochila* from the same region display the opposite pattern—overall size increases

with increasing aridity, ostensibly to reduce water loss by decreasing surface-area to volume ratios (Nevo et al. 1983).

Alternatively, results by Schmidt-Nielsen et al. (1971) show that *Sphincterochila* keeps from overheating while withdrawn during the day by maintaining a thermally insulating airspace between its soft tissue and the ground; a larger shell could mean a larger airspace, the better to avoid increased heat stress. The area where USNM 361966 was collected is close to 300 meters higher than the Monclova area, so one would expect the local environment for USNM 361966 to be somewhat cooler than that of *H. picta.* The desert snail *Xerocrassa* does conform to Bergmann's Rule (Nevo et al. 1981), but its depressed, almost trochiform shape and *Holospira picta*'s oblong, cylindro-conic shape could hardly be more different, and parallels should be drawn with caution.

It is tempting to assume that the smaller aperture size of USNM 361966 is a result of evaporation driving selection pressures, but without more specific collection localities or a rediscovery of the populations it is impossible to be sure. South and east of Monclova runs a ridge of small mountains, whose southwestern slopes are not quite as arid as the plains surrounding the city of Monclova to the north and west. USNM 361966 would likely have been collected in this area "at an altitude of 3000 feet, about halfway between Nuevo Leon and Monclova." Given the vague locality for *H. picta* "east of Monclova" (Bartsch 1925) it is difficult to be certain whether or not *H. picta* is from an ecologically similar area or a more arid climatic area.

Thompson (1974) writes of a similar situation in which two holospirids are very similar but geographically and morphologically distinct, "Because of the isolation of the two taxa from each other and because of the absence of intergrading populations, I treat them as

separate species though their interrelationship is close." He tempers this statement by prefacing it with "Cogent arguments can be given for recognizing [the populations] as distinct species or as subspecies." No intergrading populations between *H. picta* and USNM 361966 have been reported, despite recent searches.

The ANOSIMs conducted indicate that all three populations are statistically distinct from each other; that differences between groups are significantly greater than differences within groups. None of the notches of the individual populations' box-and-whisker plots in Figure 3 overlap on the Y-axis—had there been an overlap, it would have indicated the two populations were not distinct. While the R-value in the post-hoc test comparing *H. picta* and USNM 361966 was extremely small, only 0.098, it was still significantly greater than zero.

Similarly, the ADONIS analysis showed that the population to which a specimen was assigned was a significant factor in explaining the variation between shells. Even when comparing *H. picta* and USNM 361966, in which case the assigned population only explained 15.3% of the variance, it was nonetheless a significant factor. Most of the remaining variance can be attributed to the relatively wider columellas and smaller lamellar rotations of USNM 361966, combined with proportional differences due to total length (e.g. *H. picta* had a greater absolute diameter and 2nd whorl height than USNM 361966, but a smaller ratios of those measurements to total length).

The NMDS plots (Figures 4, 6, and 7) show that axial lamella rotation is one of the greatest factors in separating the three populations, and also the greatest contributor to MDS1 (Table 5). The greater the rotation of the axial lamella, the further to the left on the plot a point was placed. Height of the axial lamella contributed to both MDS1 and MDS2, with greater adjusted heights tending towards the lower left-hand corner of the graph. The greater

average lengths of *H. picta* coupled with their short axial lamellas led to the 'adjaxht' parameter (adjusted axial lamella height) being very small in this population. This contributed to the placement of many of them in the upper half of the plot.

Both the presence of a basal lamella and the presence of a parietal lamella contribute strongly to both axes. This was to be expected (Clarke 1993), as these parameters were uniformly present in one species and absent in the other two populations; hence the complete overlap of the vectors—and labels—of the parameters 'presbas' and 'pressup' in Figure 6. Their presence in a specimen tends to give it a placement on the NMDS plot to the left of and downward from the origin, which helps explain why no *Holospira monclovana* specimen was greater than 0.12 on the MDS1 axis or greater than 0 on the MDS2 axis. Adjusted internal columellar width and adjusted maximum diameter also weigh down and to the left. Adjusted columellar height, conversely, weighs up and to the right. The average ratios of columella height to total height tend to be greater in USNM 361966 and *H. picta* (~0.86 and ~0.85 respectively) than in *H. monclovana* (~0.83), which helps explain the presence of the former in the upper right quadrant.

The outlier of *Holospira monclovana,* Specimen 13, is characterized by an anomalously small axial lamellar rotation of only 116° coupled with a relatively large total length, a relatively short columella, a greater-than average axial height, and a taller-than-average second whorl. All of these factors contributed to its placement down and to the right from the mass of its conspecifics on the NMDS plot.

The upper outliers and the leftmost outliers of *H. picta* have differing explanations. Specimen 27 is characterized by a very narrow columella and an unusually short second whorl; these factors contribute to place Specimen 27 in the uppermost position on the plot.

While both its total height and its columellar height are slightly below average, the ratio of the two is higher than that of most other *H. picta* specimens, placing it further up on the NMDS plot than its conspecifics. Specimens 51 (the holotype of *H. picta*) and 25, by contrast, have unusually high values for almost all measurements; the large total length combined with large axial lamella measurements and small ratios of second whorl height place these specimens in the center left, close to *H. monclovana* specimens.

Unusually small axial lamellar rotation in conjunction with relatively short columellas and generally short total heights accounts for the extreme lower-right placement of Specimens 63, 67, and 69 from USNM 361966. Interestingly, Specimen 64 is one of the smallest shells by all measurements, but its proportions keep it close to zero on MDS2 and only combine to shift it right on MDS1.

The confidence ellipses of the NMDS plot present a quandary. Both the 99% and 95% confidence ellipses for *Holospira monclovana* are well-separated from those of the other populations, supporting the conclusion that it is a distinct species (indeed, in a distinct subgenus) compared to the other two. The 95% confidence interval ellipses of *H. picta* and USNM 361966 do not overlap, implying distinct categories, but the 99% confidence ellipses do, indicating that the null hypothesis cannot be rejected with complete certainty (Figure 7). The 95% confidence ellipses cannot be taken at face value; as this investigation includes multiple comparisons, there is a risk of inflated Type I error. The conservative Bonferroni correction for confidence level gives 98.3%—much closer to 99% than to 95% —as a coinfidence interval sufficient to maintain α=0.05 for three categories (Abdi et al. 2009).

As NMDS attempts to represent multidimensional data two-dimensionally, overlaps can sometimes be the result of projection artifacts. The risk of this is especially great in cases

where the NMDS axes fail to account for part of the data (Abdi et al. 2009), but the high R^2 values of the Shepard stress plot (Figure 5) indicate that very little variation is unaccounted for in the NMDS plot (Clarke 1993).

CONCLUSIONS

This investigation supports Bartsch's assignment (1925) of *Holospira monclovana* to the subgenus *Holospira* and confirms that both *Holospira picta* and USNM 361966 belong to another subgenus. Current taxonomic weighing indicates that the proper subgenus for these populations is *Bostrichocentrum*. These conclusions fail to support Thompson's (2011) designation of the Mexican "*Eudistemma*" as a synonym for *Holospira* s.s.

All statistical analyses confirmed a significant difference between *H. picta* and USNM 361966, but the NMDS ordination plot did show some slight overlap at the 99% confidence ellipses. Given the great distance (by holospirid standards) yet overall similarity between the two populations, it is likely that they were once subpopulations of a widespread distribution which was fragmented into two and isolated over time. This evolutionary pattern is not infrequent in the genus (Thompson 1974), and certainly holospirids were once more widely distributed, with late Cretaceous specimens being found as far north as Canada (Tozer 1956). By the most conservative estimation, USNM 361966 is a small montane variant of *Holospira (Bostrichocentrum) picta.* The confidence ellipses do not, in this analysis, justify the elevation of USNM 361966 to its own species, but the ANOSIM and ADONIS results indicate a greater separation than mere 'variant populations'—something on the level of a subspecies at the least.

It is the conclusion of this investigation that *Holospira picta* and the subspecies of *Holospira picta* that is USNM 361966 be reassigned to the subgenus *Bostrichocentrum.*

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APPENDIX A

Results of Analysis of Similarities performed using R 2.14.1

Comparing *Holospira monclovana* holotype and paratype series, *H. picta* holotype and paratype series, and USNM 361966

Dissimilarity: bray

ANOSIM statistic R: 0.3977

Significance: 0.001

Based on 999 permutations

Empirical upper confidence limits of R:

90%	95%	97.5%	99%
0.0260	0.0377	0.0477	0.0569

Dissimilarity ranks between and within classes:

	0%	25%	50%	75%	100%	N
Between	2	970.5	1767	2385.75	2926	1974
A	9	438.0	986	1540.50	2527	351
B	1	757.0	1366	1988.00	2876	325
X	37	389.0	671	1091.50	2230	276

st.diss, grouping = csv.test$type)

Comparing *H. picta* holotype and paratype series and USNM 361966

Dissimilarity: bray

ANOSIM statistic R: 0.09805

Significance: 0.006

Based on 999 permutations

Empirical upper confidence limits of R:

90%	95%	97.5%	99%
0.0358	0.0475	0.0650	0.0813

Comparing *H. monclovana* holotype and paratype series and *H. picta* holotype and paratype series

Dissimilarity: bray

ANOSIM statistic R: 0.466

Significance: 0.001

Based on 999 permutations

Empirical upper confidence limits of R:

90%	95%	97.5%	99%
0.0367	0.0550	0.0768	0.1105

Comparing *H. monclovana* holotype and paratype series and USNM 361966

Dissimilarity: bray

ANOSIM statistic R: 0.6119

Significance: 0.001

Based on 999 permutations

Empirical upper confidence limits of R:

90%	95%	97.5%	99%
0.0332	0.0535	0.0674	0.1005

APPENDIX D

Results of ADONIS (robust non-parametric MANOVA analogue) performed using R 2.4.1

adonis(formula = test.diss ~ type, data = csv.test, permutations = 999, method = "bray")

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
type	2	1.3278	0.66392	37.842	0.50563	0.001 ***
Residuals	74	1.2983	0.01754	0.49437		
Total	76	2.6261	1.00000			

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Comparing *H. picta* holotype and paratype series and USNM 361966

adonis(formula = test.dissAB ~ type, data = csv.testAB, permutations = 9999, method = "bray")

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
type	1	0.19794	0.197945	9.2204	0.15311	0.0016 **
Residuals	51	1.09487	0.021468	0.84689		
Total	52	1.29282	1.00000			

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1